



**University of
Zurich**^{UZH}

**Zurich Open Repository and
Archive**

University of Zurich
University Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2012

Dissection of Drosophila MTF-1 reveals a domain for differential target gene activation upon copper overload vs. copper starvation

Günther, V ; Waldvogel, D ; Nosswitz, M ; Georgiev, O ; Schaffner, W

Abstract: Metal-responsive transcription factor-1 (MTF-1) is a zinc finger protein conserved from mammals to insects. It mediates protection against heavy metal load by activating the expression of metallothionein and other genes. In *Drosophila*, MTF-1 serves a dual function in that it not only helps to protect against heavy metal load but also induces the expression of Ctr1B, the gene for an intestinal copper importer, upon copper starvation. By dissecting *Drosophila* MTF-1 function, we have identified determinants for nuclear import and export, and characterized a phosphorylation site mutant (T127A) that differentially affects MTF-1 target genes. Further, by generating a series of fusion proteins with the heterologous DNA binding domain of Gal4 we identified a strong, constitutive activation domain in the central region of MTF-1 (aa 352-540). By contrast, an extended fusion protein that includes MTF-1's C-terminus (aa 352-791) is not active in standard conditions but induced by copper load. The paramount regulatory importance of the C-terminal part, that harbors a cysteine-rich "metallothionein-like" domain, was corroborated by different experiments. Transgenic flies expressing C-terminally truncated MTF-1 variants displayed high constitutive transcription of both, the genes for metallothioneins and the copper importer Ctr1B. The indiscriminate activation of these genes that are normally induced under opposite conditions of copper load and copper starvation manifested itself in a shortened life span, crippled wings, and female sterility.

DOI: <https://doi.org/10.1016/j.biocel.2011.11.016>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-53025>

Journal Article

Accepted Version

Originally published at:

Günther, V; Waldvogel, D; Nosswitz, M; Georgiev, O; Schaffner, W (2012). Dissection of *Drosophila* MTF-1 reveals a domain for differential target gene activation upon copper overload vs. copper starvation. *International Journal of Biochemistry Cell Biology*, 44(2):404-411.

DOI: <https://doi.org/10.1016/j.biocel.2011.11.016>

**Dissection of *Drosophila* MTF-1 reveals a domain for differential target gene
activation upon copper overload vs. copper starvation**

Viola Günther, Dominique Waldvogel, Michael Nosswitz, Oleg Georgiev, Walter
Schaffner

From the Institute of Molecular Life Sciences, University of Zürich, Winterthurerstrasse
190, CH-8057 Zürich, Switzerland.

Correspondence to:

Prof. Dr. Walter Schaffner, Institute of Molecular Life Sciences, University of Zürich,
Winterthurerstrasse 190, CH-8057 Zürich, Switzerland;

phone: +41 44 6353150; fax: +41 44 6356811

Email: walter.schaffner@imls.uzh.ch

Abstract

Metal-responsive transcription factor-1 (MTF-1) is a zinc finger protein conserved from mammals to insects. It mediates protection against heavy metal load by activating the expression of metallothionein and other genes. In *Drosophila*, MTF-1 serves a dual function in that it not only helps to protect against heavy metal load but also induces the expression of *Ctr1B*, the gene for an intestinal copper importer, upon copper starvation. By dissecting *Drosophila* MTF-1 function, we have identified determinants for nuclear import and export, and characterized a phosphorylation site mutant (T127A) that differentially affects MTF-1 target genes. Further, by generating a series of fusion proteins with the heterologous DNA binding domain of Gal4 we identified a strong, constitutive activation domain in the central region of MTF-1 (aa 352-540). By contrast, an extended fusion protein that includes MTF-1's C-terminus (aa 352-791) is not active in standard conditions but induced by copper load. The paramount regulatory importance of the C-terminal part, that harbors a cysteine-rich "metallothionein-like" domain, was corroborated by different experiments. Transgenic flies expressing C-terminally truncated MTF-1 variants displayed high constitutive transcription of both, the genes for metallothioneins and the copper importer *Ctr1B*. The indiscriminate activation of these genes that are normally induced under opposite conditions of copper load and copper starvation manifested itself in a shortened life span, crippled wings, and female sterility.

Keywords: Metal-responsive transcription factor-1; *Drosophila*; metallothioneins; transcriptional regulation; heavy metal homeostasis.

1. Introduction

A key player in heavy metal homeostasis is the metal-responsive transcription factor-1 (MTF-1, also termed metal-regulatory transcription factor-1 or metal response element-binding transcription factor-1). MTF-1 is activated by heavy metal load (Heuchel et al., 1994; Westin and Schaffner, 1988) and drives the transcription of metallothioneins and other genes involved in heavy metal homeostasis. Metallothioneins are small, cysteine-rich proteins that protect cells from heavy metal load and oxidative stress. MTF-1 has been isolated and characterized in a diverse set of organisms ranging from the fruit fly *Drosophila melanogaster* (Zhang et al., 2001) to mammals, including mice (Radtke et al., 1993) and humans (Brugnera et al., 1994; Otsuka et al., 1994). While the DNA-binding zinc finger region of human and *Drosophila* MTF-1 is conserved, there is little, if any conservation of protein sequence outside of the zinc fingers (Zhang et al., 2001). Differences in the function and regulation of human and *Drosophila* MTF-1 became evident early on. Mammalian MTF-1 strongly induces transcription of target genes in response to zinc and cadmium, whereas the *Drosophila* homolog is best activated by copper and cadmium. Additionally, *Drosophila* MTF-1 has a dual role: On the one hand it activates transcription of the genes for metallothioneins and the copper transporter *DmATP7* under dietary copper overload (Burke et al., 2008; Zhang et al., 2001), on the other hand under low copper conditions it activates expression of the copper importer *Ctr1B* to protect flies from copper starvation (Selvaraj et al., 2005). Flies without *MTF-1* develop normally under standard food conditions, but cannot survive heavy metal stress (Egli et al., 2003). In contrast to this in mice, knockout of *MTF-1* is lethal and embryos die at day 14 of gestation (Günes et al., 1998). A motif rich in cysteines (“cysteine cluster”) is present in both mammalian and *Drosophila* MTF-1, but displays different functions. The cysteine cluster of *Drosophila* MTF-1

⁵⁴⁷CNCTNCKCDQTKSCHGGDC⁵⁶⁵ is important for sensing excess intracellular copper (Chen et al., 2008). Mutant flies missing this cysteine cluster fail to activate metallothioneins and show an impaired survival in response to high copper (Chen et al., 2008). The mammalian cysteine cluster (⁶³²CQCQCAC⁶³⁸ in human MTF-1) is essential for the transcriptional activity of MTF-1 (Chen et al., 2004). In human MTF-1, specific sequence motifs for nuclear localization, nuclear export and transcriptional activation have been identified (Lindert et al., 2009; Radtke et al., 1995; Saydam et al., 2001). MTF-1's metal inducibility localizes to the zinc finger domain, which shows a relatively low affinity for zinc (Heuchel et al., 1994; Otsuka et al., 2000), and a metal-sensing acidic transactivation domain (Lindert et al., 2009). Here we have characterized a number of *Drosophila* MTF-1 mutants in order to identify functional elements in this protein. We determined the domains that regulate the cellular distribution of the protein, analyzed the effect of phosphorylation site mutations in zinc fingers 1 and 2, and defined a constitutively active acidic transactivation domain. Finally we show that the C-terminal region, which is highly conserved among insects, besides having nuclear export function, negatively regulates transcription, in part by downregulating MTF-1 protein levels. Most importantly, this domain is essential to discriminate between excess and scarcity of copper supply.

2. Materials and Methods

2.1. Cell culture and transfections

Drosophila S2 and HEK293T cells were cultured as described previously (Lindert et al., 2009; Zhang et al., 2001). Cells were transfected with 2 µg total DNA per ml cell culture

medium (adjusted with Herring sperm DNA) by the calcium phosphate co-precipitation method.

2.2. Plasmid constructions

Potential NES sequences were cloned as oligonucleotides in a vector containing tandem repeated EGFP (a gift of Dr. Ulrike Kutay, ETHZ, Switzerland). Constructs used for testing NLS function were made by cloning MTF-1 fragments amplified by PCR into a plasmid expressing the Myc-tagged chicken pyruvate kinase (Myc-PK) (Siomi and Dreyfuss, 1995). Gal4 fusion constructs are based on *pAc5/V5-HisB* (Invitrogen) containing the *Gal4* DNA-binding domain (amino acids 1-147) and PCR amplified fragments of *Drosophila MTF-1*. The *2xGal-OVEC* reporter construct was described before (Seipel et al., 1992). The *Actin 5c-OVEC* reference plasmid was made by introducing the fragment of *pAc5/V5-HisB* comprising the 2.7 kb *Actin 5c* promoter into *OVEC-ref* (Westin et al., 1987). The *Drosophila* transformation vectors were generated by cloning the *Drosophila MTF-1* cDNA under the control of the *Tubulin* promoter into *pAttB* (Bischof et al., 2007) and mutations were introduced via site-directed mutagenesis (QuikChange, Stratagene). Detailed information about oligonucleotide sequences and cloning strategies are available on request.

2.3. Indirect immunofluorescence

HEK293T cells, grown on cover slips in a 12-well tissue culture plate, were transfected with *2xEGFP-NES* or *Myc-PK-NLS* fusion constructs. Immunofluorescent staining was performed as described previously (Lindert et al., 2009; Saydam et al., 2001).

2.4. Fly stock keeping and generation of transgenic flies

Flies were maintained as described previously (Hua et al., 2011). *Drosophila MTF-1* transgenes were introduced into the fly genome at an *AttP* landing site located at genomic position 51D using the phiC31 integration system (Bischof et al., 2007) and flies were crossed to a *MTF-1*^{-/-} background (except for deletion constructs, which displayed a dominant effect and had a reduced fitness in a *MTF-1*^{-/-} background so that stocks could not be maintained). Experiments were performed with flies that carried one copy of the transgene. To measure eclosion rates, five transgenic males were crossed to four *y w* virgins. For the longevity assay approx. 100 freshly eclosed female flies were put on normal food in groups of 10, counted every 1-2 days and transferred to fresh food every 3-4 days.

2.5. S1 nuclease protection assay

RNA of 30-40 3rd instar larvae or freshly eclosed flies, raised on either standard food or food with the indicated supplements, was isolated using TRIzol (Life Technologies) followed by the S1 nuclease mapping of transcripts performed as described previously (Weaver and Weissmann, 1979). mRNA levels of the *Actin 5c* gene were used for normalization of *MtnA*, *MtnB* and *Ctr1B* transcripts. For testing the Gal4 DNA-binding domain fusion constructs 1,4x10⁷ S2 cells were transfected with 7 µg of an 2xUAS-*β-globin* reporter plasmid, 3.5 µg of an *Actin 5c-β-globin* reference plasmid and 35 ng of the *Gal4* fusion construct. RNA was isolated by phenol/DCM extraction and subjected to S1 nuclease protection assay (Weaver and Weissmann, 1979; Westin et al., 1987). Standard deviations of three independent experiments were calculated.

2.6. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts of transiently transfected S2 cells were prepared as described previously (Schreiber et al., 1989). 30 µg of nuclear protein and approx. 40 fmol of end-labeled MRE-s double-stranded oligonucleotide were used in a binding reaction according to (Radtke et al., 1993). Samples were separated on a 4% polyacrylamide gel in 0.25x TBE buffer.

2.7. Western blot analysis of whole cell extracts and nuclear extracts

S2 cells were transfected with the respective *MTF-1* expression plasmids. Whole cell extracts were prepared in a buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 8, 5 mM EDTA, 0.5 % NP-40, 1 mM DTT and protease inhibitors. Whole cell or nuclear extracts were loaded on a 6 % tris-glycine SDS-PAGE and transferred on a PVDF-membrane (Amersham). MTF-1 was detected using a 1:2500 dilution of a polyclonal rabbit antibody raised against aa 406-540 of *Drosophila* MTF-1 (a kind gift of Dr. Michael Marr, Brandeis University, Massachusetts), followed by a HRP-conjugate anti-rabbit IgG at 1:5000 dilution (GE Healthcare, NA934). Proteins were visualized using the ECL chemiluminescent detection system (Pierce).

3. Results

3.1. *Drosophila* MTF-1 encodes two segments mediating nuclear export and the main nuclear import function is localized to zinc fingers 1-3.

We screened the *Drosophila* sequence for motifs that could mediate nuclear export. Nuclear export signals (NES) of human MTF-1 show the typical leucine-rich sequence, while in *Drosophila* MTF-1 we could only identify possible NES motifs with a more

relaxed requirement (Figure 1A and B) in which other hydrophobic amino acids, namely Ile, Val, Phe, Met, can substitute for leucine (Kutay and Güttinger, 2005). To study *Drosophila* NES sequences, mammalian cells were used, because they are larger and allow for ready distinction between nuclear and cytoplasmic staining (Bi et al., 2005; Murai et al., 2003). Each of the three candidate motifs was fused to a reporter protein consisting of two tandem copies of fluorescent protein (2xEGFP) and expressed in HEK293T cells to visualize nuclear export (Figure 1C). Two of the motifs, NES1 and NES2, induced cytoplasmic localization of the 2xEGFP-fusion protein. Both these motifs show no obvious sequence homology to the human MTF-1 NES, but we note that the sequence of NES2 is highly conserved among insects (Figure 1D) and possibly contains a protein destabilizing signal, as a longer exposure time had to be chosen to visualize the NES2-2xEGFP fusion protein.

In search for nuclear localization signals (NLS), most of the MTF-1 coding region was divided into subsegments (Figure 1A) which were individually tested upon fusion to Myc-PK, a reporter protein moiety that is exclusively cytoplasmic, unless linked to a nuclear localization signal (Siomi and Dreyfuss, 1995). Here there was a good congruence with mammalian MTF-1 in that the major NLS also localized to a broad region of zinc fingers 1-3 (segment b, Figure 1E). Other segments localized in the zinc finger region had weaker or no activity (segments c or d, respectively). Additionally, a weak nuclear localization activity was associated with a segment rich in basic amino acids downstream of the zinc finger region (segment e) and with a subsegment of the transactivation domain (segment g). The activity of the latter might be an indirect one, brought about via an interaction with a cofactor of transcriptional activation.

185 3.2. *MTF-1* phosphorylation sites discriminate between different copper-inducible target
186 genes.

187 In the course of the *Drosophila* PhosphoPep project, in which the whole proteome of
188 *Drosophila* Kc167 cells was screened for phosphorylated residues (Bodenmiller et al.,
189 2008), four specific sites in MTF-1 were shown to carry phosphate groups, namely
190 Tyr118, Ser126, Thr127 and Ser160. To explore the role of these sites in *Drosophila*
191 MTF-1, individual residues were changed to alanines and the modified transgenes
192 were introduced into the *Drosophila* genome in a *MTF-1* null mutant background.
193 Expression levels were determined for three well-established target genes of MTF-1:
194 two metallothioneins (*MtnA* and *MtnB*) and the copper importer *Ctr1B*. To measure
195 transcript levels, RNA from 3rd instar larvae grown on normal food, or food
196 supplemented with either copper, cadmium or the copper chelator BCS (bathocuproine
197 disulfonate) was analyzed (Figure 2A). Interestingly, the two metallothionein genes
198 responded differently to the phosphorylation site mutants. Whereas *MtnA* was only
199 mildly affected, expression of *MtnB* was strongly impaired by three of the mutations,
200 especially by the T127A mutation. By contrast, S160A tended to increase both basal
201 and copper induced expression. Upon cadmium load wild type MTF-1 induced the
202 expression of *MtnB*, but not *MtnA*, whereas the T127A mutant induced *MtnA*
203 expression and the S160A mutant lost the ability to activate *MtnB*. Regulation of the
204 copper importer *Ctr1B*, whose transcripts are substantially induced upon copper
205 starvation, was hardly affected by these mutations. In parallel, the C-terminal domain
206 was mutagenized, which, due to its high cysteine content, had been dubbed a
207 metallothionein-like domain (Chen et al., 2008). All 18 cysteines between amino acids
208 642 and 766 were substituted by alanines (mcysCTD in figures). This modification did
209 not affect basal level expression of target genes, but substantially reduced the

expression of *MtnA* and *MtnB* upon copper (and cadmium) load, bringing metal-responsive transcription close to basal level. To see whether the mutagenesis of the phosphorylation sites and the cysteines affected the DNA-binding activity of MTF-1, nuclear extracts of transfected S2 cells were subjected to EMSA, using a consensus binding site for MTF-1 (metal response element, MRE-s (Radtke et al., 1993)). Only the S160A mutant, which also yielded an elevated activity in the transcription assay, showed a somewhat stronger EMSA band whereas the other mutants produced bands of about the same intensity (Figure 2B). Thus the lower transcriptional activity of some of the mutants is not merely due to a reduced DNA-binding ability.

3.3. C-terminal deletions of Drosophila MTF-1 lose the ability to discriminate between copper load and copper starvation.

To further identify regions in MTF-1 that regulate its function, we tested three shortened versions, namely aa 1-579 (Δ 580-791), aa 1-636 (Δ 637-791) and aa 1-760 (Δ 761-791). These were transfected into S2 cells and their DNA-binding ability assayed by EMSA of nuclear extracts (Figure 2B). All three truncations yielded a considerably stronger shift than wild type MTF-1 or the phosphorylation site mutations, suggesting that an inhibitory domain lies in the C-terminal segment. Furthermore, there was no decrease in DNA-binding activity for the truncation mutants after four hours of copper induction, as observed for wild type MTF-1. The higher DNA-binding activity of the Δ 761-791 truncation in untreated cells is not due to higher protein levels as judged by western blot of nuclear and whole cell extracts (Figure 3A). Furthermore, in line with a destabilizing function of the C-terminal 30 amino acids (see Figure 1D) the Δ 761-791 mutant protein, unlike the wild type protein, is not degraded after prolonged copper treatment (Figure 3A).

In flies expression of the truncation mutants, even on a wild type *MTF-1* background, had dramatic effects. Females transgenic for $\Delta 580-791$ and $\Delta 637-791$ were sterile (data not shown) and the wing structure was distorted in both males and females. While the short deletion $\Delta 761-791$ only had “notched” wings, the two bigger deletions caused severely malformed, crumpled wings (Figure 3B). Additionally, flies expressing the truncations had an obviously reduced vitality, which was measured in two ways. First, the development to adult flies was monitored in a cross between males expressing the truncation mutant and *y w* virgins (Figure 3C). While the transgenic lines expressing the short deletion had eclosure rates approaching those of control flies (transgenic flies carrying a wild type *MTF-1* gene), those with bigger deletions showed a substantially reduced eclosure rate, with less than half of the embryos developing to adulthood. Different food compositions (normal food (NF), copper or zinc supplemented or copper depleted food) did not significantly affect eclosure. If anything, zinc exerted a negative effect on the vitality of flies expressing a truncation mutant (Figure 3C). Second, *MTF-1* truncation transgenes also reduced the lifespan of adult flies, with a negative correlation between life span and the extent of the C-terminal deletion. Whereas control flies and flies with a wild type *MTF-1* transgene had similar lifespans (median lifespan 49 and 47 days, respectively, Figure 3D), increasing the size of the truncation strongly decreased the lifespan to a median of 28, 13 and 10 days for the $\Delta 761-791$, $\Delta 637-791$ and $\Delta 580-791$ variants, respectively.

The most striking effect was however seen when the expression of the major target genes *MtnA*, *MtnB* and *Ctr1B* was tested. Transcript levels were measured in freshly eclosed flies that had been raised on standard food. Here the truncated MTF-1 forms were unable to properly regulate the target genes, whereby the severity of the dysregulation increased with the extent of the truncation; both *MtnA* and *MtnB* and

surprisingly also *Ctr1B* were strongly overexpressed (Figure 3E). These high expression levels of MTF-1 target genes were only slightly altered by manipulation of the ambient copper concentration by food supplementation with copper or BCS (data not shown).

To determine the interplay of the MTF-1 truncations with their major target genes, we tested two of them in a genetic background of *Ctr1B* heterozygosity or complete knockout. Loss of one copy of *Ctr1B* ameliorated the wing phenotype (Figure 4A, left panel) and a homozygous knockout of *Ctr1B* rescued the wing phenotype almost completely, whereby the 5th longitudinal vein tended to be incomplete (Figure 4A, middle panel; lifespan was however not restored (see below)). In marked contrast to this, combination with a knockout of four of the five *Drosophila* metallothionein genes (*Mtn A, B, C, D*, termed quadruple or *qMtn* mutant (Egli et al., 2006)) exacerbated the wing phenotype in that the wings were reduced to rudiments (Figure 4A, right panel). The wing effect is not easily explainable since curiously, the wing structure could be rescued not only by elimination of *Ctr1B*, which decreases body copper levels, but also by dietary copper supplement (data not shown), indicating specific effects depending on tissue and/or developmental stage.

As noted above, elimination of *Ctr1B* had little if any effect on the lifespan (Figure 4B): *Ctr1B* knockout flies without or with the wild type *MTF-1* transgene showed a median lifespan of 51 or 50 days, respectively, whereas the median survival of the $\Delta 637-791$ and $\Delta 580-791$ flies was still reduced to 21 and 18 days, respectively. In order to find out whether the upregulation of metallothioneins by the MTF-1 truncations was primarily due to higher copper import resulting from *Ctr1B* hyperinduction, the transcripts of *MtnA* and *MtnB* were determined in the *Ctr1B* knockout background. As shown in Figure 4C, the two genes were overexpressed even in the absence of *Ctr1B*.

We were not able to test *Ctr1B* expression in the context of these truncation mutants in a quadruple metallothionein knockout background (*qMtn*), as *qMtn* heterozygous males carrying the truncations showed a severely reduced fertility. Moreover, eclosure rates were very low for flies carrying the truncations in a *qMtn* homozygous background (Figure 4D), and these flies could not be crossed further.

3.4. The acidic activation domain of *Drosophila* MTF-1, spanning amino acids 352 to 540, is constitutively active.

The above results had suggested that the C-terminal segment of MTF-1 inhibits a constitutive transcriptional activation domain. In search for this putative domain we fused extended C-terminal variants of MTF-1 to the DNA-binding domain of the heterologous yeast transcription factor Gal4. Various fusion proteins (Figure 5A) were tested in S2 cells for their ability to drive transcription of a *β -globin* reporter under the control of a *2xUAS* promoter. The construct carrying aa 286-791 of *Drosophila* MTF-1 (segment a) showed a low basal activity that was strongly induced by 100 μ M copper or 10 μ M cadmium (Figure 5B). As had been shown before in the context of full length MTF-1 (Chen et al., 2008), mutation of the cysteine cluster (segment b) eliminated copper induced activity, but a partial response to cadmium was maintained. As for intact MTF-1 and its truncations (Figure 3E), the C-terminal deletion in the Gal4-fusion (segment c) resulted in increased basal activity combined with a decreased inducibility. A very strong and constitutive activation was observed with segment i (aa 352-540). Like the situation in mammalian MTF-1, this segment is also located downstream of the zinc fingers (Figure 6). However, its primary sequence is different, and unlike the mammalian domain it is not by itself responsive to metals.

309

310 **4. Discussion**

311 Human and *Drosophila* MTF-1 share a high protein sequence similarity in their zinc
312 finger region (81 %), whereas little similarity is present outside of the zinc fingers
313 (<23 %). In this context it was surprising that *Drosophila* MTF-1 could largely cross-
314 complement its ortholog in mammalian cells lacking MTF-1 and vice versa
315 (Balamurugan et al., 2004). In order to further elucidate similarities and differences
316 between the two proteins we have identified a number of novel features of regulatory
317 importance in *Drosophila* MTF-1. We find that the general functions of human and
318 *Drosophila* MTF-1 are very similar but the location of functional domains within the
319 protein and their primary sequences are mostly different (Figure 6).

320 In contrast to human MTF-1, whose isolated activation domain retains considerable
321 metal-responsiveness, metal-sensing in *Drosophila* MTF-1 is separable from its
322 activation domain. Phosphorylation is widely exploited to regulate cellular proteins,
323 including transcription factors. Phosphorylation has also been implicated in the
324 regulation of mammalian MTF-1, but no specific amino acid residue was shown to be
325 critical (LaRochelle et al., 2001; Saydam et al., 2002). In a phosphoproteome screen
326 positions Tyr118, Ser126, Thr127 and Ser160 of *Drosophila* MTF-1 were found to be
327 phosphorylated (Bodenmiller et al., 2008). Our results indicate these sites are involved
328 in target gene discrimination: expression of *MtnB*, far more than the expression of *MtnA*
329 and *Ctr1B*, is affected in phosphorylation site mutants. This is the first time that mutants
330 of MTF-1 were found that discriminate between individual metallothionein genes. The
331 promoters of the *MtnA* and *MtnB* genes were shown before to require an interaction of
332 different transcriptional co-activators with MTF-1 (Marr et al., 2006), thus the

333 phosphorylation sites investigated here might be critical to allow for these promoter-
334 specific interactions.

335 The C-terminal domain of MTF-1 (aa 657-791) is rich in cysteines and thus was
336 referred to as metallothionein-like domain (Chen et al., 2008). The mutant in which
337 those cysteines were replaced by alanines (mcysCTD) failed to activate *MtnA* and
338 *MtnB* genes under copper load. These results establish the C-terminal cysteine-rich
339 region, in addition to the previously characterized more centrally located cysteine
340 cluster (aa 547-565) (Chen et al., 2008), as being important for metal induced activity.
341 Like mutation of the central cysteine cluster, mutation of the C-terminal cysteines did
342 not impair *Ctr1B* induction by copper starvation.

343 Since none of the mutations substantially affected the activity of the *Ctr1B* gene under
344 copper starvation, we propose that Ctr1B activation is a process distinct from metal
345 induced activity. A very strong binding of MTF-1 to the *Ctr1B* upstream region seems to
346 ensure that even under conditions of no metal load, this gene remains active and that
347 the downregulation upon copper overload is an active repression process (Selvaraj et
348 al., 2005). Our data show that the negative regulation of *Ctr1B* involves the C-terminal
349 domain, likely via an intramolecular repressive interaction or interaction with a
350 repressor protein, because truncations result in high *Ctr1B* transcription in the absence
351 of copper starvation.

352 Expression of C-terminal truncations of MTF-1 in transgenic flies resulted in severely
353 compromised fertility, malformed wings and reduced lifespan, likely explainable by a
354 simultaneous strong expression of metallothioneins and the copper importer *Ctr1B*,
355 which normally are only induced under conditions of copper load and copper starvation,
356 respectively. The role of copper in *Drosophila* fertility was already observed in the
357 context of a double knockout of the copper transporters *Ctr1B* and *Ctr1C*, which

renders males sterile (Steiger et al., 2010), whereas a defective wing development has not, so far, been documented for flies with a disturbed copper homeostasis. Genes determining wing structure, like *Notch*, *Wingless* and *Vestigial*, even though their proximal promoter region harbors no obvious MREs, could be direct or indirect targets of dysregulated MTF-1 function. A phenotype including female subfertility, reduced vitality and morphological defects in the wings was also observed with a knockout of the *TIMP* gene (*tissue inhibitor of metalloproteinases*) (Godenschwege et al., 2000) and we note that one of its target proteins, Mmp1, has MREs in its proximal promoter region and thus could be a target of dysregulated MTF-1 activity.

A clear difference to human MTF-1 is evident if the subcellular localization of MTF-1 is observed under metal load. While mammalian MTF-1 is mainly cytoplasmic and rapidly translocates to the nucleus upon metal stress (Saydam et al., 2001; Smirnova et al., 2000), the bulk of *Drosophila* MTF-1 was already DNA-bound under non-stress conditions and DNA-binding activity was strongly reduced 1 h after metal induction. In addition to a reduced DNA-binding activity, a fraction of the protein is degraded within 4 hrs after metal induction, likely as a part of a negative feedback regulation to prevent an overshooting reaction. The last 30 amino acids of *Drosophila* MTF-1 play a critical role in this process, as the $\Delta 761-791$ mutant fully retained DNA-binding ability and protein levels stayed high upon metal treatment, indicating a negative regulatory function of the C-terminus. Taken together, our data reveal the paramount importance of the C-terminal region including not only the cysteine cluster, but also the metallothionein-like domain downstream of it, for proper functioning of *Drosophila* MTF-1 in heavy metal homeostasis and detoxification.

Acknowledgements: We thank Dr. Michael Marr (Brandeis University, Massachusetts) for communicating results prior to publication, valuable discussions and the kind gift of anti-dMTF-1 antibody, Drs. Ulrike Kutay (ETH Zürich) and Gideon Dreyfuss (University of Pennsylvania School of Medicine) for the gifts of the 2xEGFP and Myc-PK plasmids, respectively, Drs. Johannes Bischof and Konrad Basler for the AttB-51D fly line and Dr. George Hausmann for critical reading of the manuscript. This work was supported by the Swiss National Science Foundation and the Kanton Zürich.

Abbreviations: aa, amino acid; BCS, bathocuproine sulfonate; Ctr1B, copper transporter 1B; EGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility shift assay; MTF-1, metal-responsive transcription factor-1; Mtn, metallothionein; NES, nuclear export signal; NLS, nuclear localization signal; OVEC, oligonucleotide vector; qMtn, quadruple metallothionein knockout.

References

- Balamurugan K, Egli D, Selvaraj A, Zhang B, Georgiev O, Schaffner W. Metal-responsive transcription factor (MTF-1) and heavy metal stress response in *Drosophila* and mammalian cells: a functional comparison. *Biol Chem* 2004; 385: 597-603.
- Bi X, Jones T, Abbasi F, Lee H, Stultz B, Hursh DA, et al. *Drosophila caliban*, a nuclear export mediator, can function as a tumor suppressor in human lung cancer cells. *Oncogene* 2005; 24: 8229-39.
- Bischof J, Maeda RK, Hediger M, Karch F, Basler K. An optimized transgenesis system for *Drosophila* using germ-line-specific phiC31 integrases. *Proc Natl Acad Sci U.S.A* 2007; 104: 3312-7.
- Bodenmiller B, Campbell D, Gerrits B, Lam H, Jovanovic M, Picotti P, et al. PhosphoPep--a database of protein phosphorylation sites in model organisms. *Nat Biotechnol* 2008; 26: 1339-40.

409 Brugnera E, Georgiev O, Radtke F, Heuchel R, Baker E, Sutherland GR, et al. Cloning,
410 chromosomal mapping and characterization of the human metal-regulatory
411 transcription factor MTF-1. *Nucleic Acids Res* 1994; 22: 3167-73.

412 Burke R, Commons E, Camakaris J. Expression and localisation of the essential
413 copper transporter DmATP7 in *Drosophila* neuronal and intestinal tissues. *Int J*
414 *Biochem Cell Biol* 2008; 40: 1850-60.

415 Chen X, Hua H, Balamurugan K, Kong X, Zhang L, George GN, et al. Copper sensing
416 function of *Drosophila* metal-responsive transcription factor-1 is mediated by a
417 tetranuclear Cu(I) cluster. *Nucleic Acids Res* 2008; 36: 3128-38.

418 Chen X, Zhang B, Harmon PM, Schaffner W, Peterson DO, Giedroc DP. A novel
419 cysteine cluster in human metal-responsive transcription factor 1 is required for heavy
420 metal-induced transcriptional activation in vivo. *J Biol Chem* 2004; 279: 4515-22.

421 Egli D, Selvaraj A, Yepiskoposyan H, Zhang B, Hafen E, Georgiev O, et al. Knockout of
422 "metal-responsive transcription factor" MTF-1 in *Drosophila* by homologous
423 recombination reveals its central role in heavy metal homeostasis. *EMBO J* 2003; 22:
424 100-8.

425 Egli D, Yepiskoposyan H, Selvaraj A, Balamurugan K, Rajaram R, Simons A, et al. A
426 family knockout of all four *Drosophila* metallothioneins reveals a central role in copper
427 homeostasis and detoxification. *Mol Cell Biol* 2006; 26: 2286-96.

428 Godenschwege TA, Pohar N, Buchner S, Buchner E. Inflated wings, tissue autolysis
429 and early death in tissue inhibitor of metalloproteinases mutants of *Drosophila*. *Eur J*
430 *Cell Biol* 2000; 79: 495-501.

431 Günes C, Heuchel R, Georgiev O, Müller KH, Lichtlen P, Blüthmann H, et al.
432 Embryonic lethality and liver degeneration in mice lacking the metal-responsive
433 transcriptional activator MTF-1. *EMBO J* 1998; 17: 2846-54.

434 Heuchel R, Radtke F, Georgiev O, Stark G, Aguet M, Schaffner W. The transcription
435 factor MTF-1 is essential for basal and heavy metal-induced metallothionein gene
436 expression. *EMBO J* 1994; 13: 2870-5.

437 Hua H, Günther V, Georgiev O, Schaffner W. Distorted copper homeostasis with
438 decreased sensitivity to cisplatin upon chaperone Atox1 deletion in *Drosophila*.
439 *Biometals* 2011; 24: 445-53.

440 Kutay U, Güttinger S. Leucine-rich nuclear-export signals: born to be weak. *Trends Cell*
441 *Biol* 2005; 15: 121-4.

442 LaRochelle O, Gagné V, Charron J, Soh JW, Séguin C. Phosphorylation is involved in
443 the activation of metal-regulatory transcription factor 1 in response to metal ions. *J Biol*
444 *Chem* 2001; 276: 41879-88.

445 Lindert U, Cramer M, Meuli M, Georgiev O, Schaffner W. Metal-responsive
 446 transcription factor 1 (MTF-1) activity is regulated by a nonconventional nuclear
 447 localization signal and a metal-responsive transactivation domain. *Mol Cell Biol* 2009;
 448 29: 6283-93.

449 Marr MT 2nd, Isogai Y, Wright KJ, Tjian R. Coactivator cross-talk specifies
 450 transcriptional output. *Genes Dev* 2006; 20: 1458-69.

451 Murai N, Murakami Y, Matsufuji S. Identification of nuclear export signals in antizyme-
 452 1. *J Biol Chem* 2003; 278: 44791-8.

453 Otsuka F, Iwamatsu A, Suzuki K, Ohsawa M, Hamer DH, Koizumi S. Purification and
 454 characterization of a protein that binds to metal responsive elements of the human
 455 metallothionein IIA gene. *J Biol Chem* 1994; 269: 23700-7.

456 Otsuka F, Okugaito I, Ohsawa M, Iwamatsu A, Suzuki K, Koizumi S. Novel responses
 457 of ZRF, a variant of human MTF-1, to in vivo treatment with heavy metals. *Biochim*
 458 *Biophys Acta* 2000; 1492: 330-40.

459 Radtke F, Georgiev O, Müller HP, Brugnera E, Schaffner W. Functional domains of the
 460 heavy metal-responsive transcription regulator MTF-1. *Nucleic Acids Res* 1995; 23:
 461 2277-86.

462 Radtke F, Heuchel R, Georgiev O, Hergersberg M, Gariglio M, Dembic Z, et al. Cloned
 463 transcription factor MTF-1 activates the mouse metallothionein I promoter. *EMBO J*
 464 1993; 12: 1355-62.

465 Saydam N, Georgiev O, Nakano MY, Greber UF, Schaffner W. Nucleo-cytoplasmic
 466 trafficking of metal-regulatory transcription factor 1 is regulated by diverse stress
 467 signals. *J Biol Chem* 2001; 276: 25487-95.

468 Saydam N, Adams TK, Steiner F, Schaffner W, Freedman JH. Regulation of
 469 metallothionein transcription by the metal-responsive transcription factor MTF-1:
 470 identification of signal transduction cascades that control metal-inducible transcription.
 471 *J Biol Chem* 2002; 277: 20438-45.

472 Schreiber E, Matthias P, Müller MM, Schaffner W. Rapid detection of octamer binding
 473 proteins with "mini-extracts", prepared from a small number of cells. *Nucleic Acids Res*
 474 1989; 17: 6419.

475 Seipel K, Georgiev O, Schaffner W. Different activation domains stimulate transcription
 476 from remote ('enhancer') and proximal ('promoter') positions. *EMBO J* 1992; 11: 4961-
 477 8.

478 Selvaraj A, Balamurugan K, Yepiskoposyan H, Zhou H, Egli D, Georgiev O, et al.
 479 Metal-responsive transcription factor (MTF-1) handles both extremes, copper load and
 480 copper starvation, by activating different genes. *Genes Dev* 2005; 19: 891-6.

481 Siomi H, Dreyfuss G. A nuclear localization domain in the hnRNP A1 protein. J Cell
 482 Biol 1995; 129: 551-60.

483 Smirnova IV, Bittel DC, Ravindra R, Jiang H, Andrews GK. Zinc and cadmium can
 484 promote rapid nuclear translocation of metal response element-binding transcription
 485 factor-1. J Biol Chem 2000; 275: 9377-84.

486 Steiger D, Fetchko M, Vardanyan A, Atanesyan L, Steiner K, Turski ML, et al. The
 487 *Drosophila* copper transporter Ctr1C functions in male fertility. J Biol Chem 2010; 285:
 488 17089-97.

489 Weaver RF, Weissmann C. Mapping of RNA by a modification of the Berk-Sharp
 490 procedure: the 5' termini of 15 S beta-globin mRNA precursor and mature 10 s beta-
 491 globin mRNA have identical map coordinates. Nucleic Acids Res 1979; 7: 1175-93.

492 Westin G, Gerster T, Müller MM, Schaffner G, Schaffner W. OVEC, a versatile system
 493 to study transcription in mammalian cells and cell-free extracts. Nucleic Acids Res
 494 1987; 15: 6787-98.

495 Westin G, Schaffner W. A zinc-responsive factor interacts with a metal-regulated
 496 enhancer element (MRE) of the mouse metallothionein-I gene. EMBO J 1988; 7: 3763-
 497 70.

498 Zhang B, Egli D, Georgiev O, Schaffner W. The *Drosophila* homolog of mammalian
 499 zinc finger factor MTF-1 activates transcription in response to heavy metals. Mol Cell
 500 Biol 2001; 21: 4505-14.

501

502 **Figure legends**

503 **Figure1: Identification of nuclear localization (NLS) and nuclear export signals**
 504 **(NES) of MTF-1.** (A) Schematic view of *Drosophila* MTF-1. Segments marked yellow
 505 with a high content of hydrophobic amino acids were tested for nuclear export function
 506 (red letters in Figure 1B). Stretches with a high content of basic amino acids that could
 507 represent a canonical nuclear localization signal (NLS), are depicted in blue. The bars
 508 a-k at the bottom show MTF-1 fragments that were tested for nuclear localization
 509 function (see Figure 1D). (C) Three potential NES sequences were tested in HEK293T
 510 cells as 2xEGFP-fusion proteins. 150 ms exposure time was chosen for all recordings,
 511 except for NES2 where an exposure time of 500 ms was required. (D) Protein

sequence alignment of the C-terminal motif of *Drosophila melanogaster* (D. mel),
Anopheles gambiae (A. gam), the parasitic wasp *Nasonia vitripennis* (N. vit), the honey
bee *Apis mellifera* (A. mel) and the flour beetle *Tribolium castaneum* (T. cas). (E)
Subsegments of MTF-1 were tested for their ability to confer nuclear localization to the
Myc-PK reporter protein. For each construct >200 cells were counted and classified in
the indicated categories: N: nuclear localization; N>C: mainly nuclear localization; N=C:
equal distribution; C>N: mainly cytoplasmic localization; C: cytoplasmic localization.

Figure 2: *Drosophila* MTF-1 phosphorylation site mutants discriminate between different target genes. (A) MTF-1 cDNA constructs containing mutations of phosphorylation sites and the mcysCTD mutant were integrated into the *Drosophila* genome and brought to a MTF-1 ^{-/-} background. Larvae were raised on the indicated food to 3rd instar stage, collected and transcript levels detected by the S1-nuclease protection assay. The copper-/BCS-induced level of wild type MTF-1 was set to 1. (B) EMSA with a synthetic MRE-containing oligonucleotide (MRE-s) and nuclear extracts of S2 cells that had been transfected with the different MTF-1 expression constructs and treated with 400 μ M CuSO₄ for 4 hrs if indicated. Note that the band positions under native EMSA gel conditions are not strictly related to the protein molecular mass. The mcysCTD mutant gave a relatively strong, blurred band that might indicate partial protein misfolding.

Figure 3: C-terminal truncation of *Drosophila* MTF-1 eliminates an autoinhibitory function and reduces vitality. (A) EMSA and western blot analyses of nuclear (NX) or whole cell extracts (WCE) of S2 cells. Cells were transfected with a wild type MTF-1 or

Δ761-791 expression plasmid and treated with 400 μM CuSO₄ prior to cell harvest for the indicated time points. For comparison extracts of non-transfected cells are shown. (B) Wings of flies transgenic for MTF-1 expression constructs in a wild type *MTF-1* background. (C) Flies heterozygous for wild type *MTF-1* or truncation mutants were crossed to *y w* flies on normal food (NF) or supplemented food as indicated and eclosure rates of transgenic flies vs. segregants lacking it were determined. Error bars indicate standard deviations from three independent crosses. (D) Shortened life span of flies expressing truncation mutants. (E) High constitutive transcription of metallothionein genes and the copper importer *Ctr1B* gene in flies expressing truncated MTF-1. mRNA levels of freshly eclosed flies that had been raised on normal food were quantified by S1 nuclease protection.

Figure 4: Wing phenotype, but not shortened life span of MTF-1 truncations, is rescued by loss of copper importer *Ctr1B*. (A) Wings of flies transgenic for truncated MTF-1 in a *Ctr1B*^{+/-}, *Ctr1B*^{-/-} or *qMtn* background. (B) Life span of truncation mutants in a *Ctr1B*^{-/-} background. (C) Metallothionein genes were still upregulated in the *Ctr1B*^{-/-} background. *MtnA* and *MtnB* mRNA levels of freshly eclosed flies raised on normal food were determined by the S1 assay. (D) Virgins (*y w* ; + ; *qMtn*) were crossed to transgenic males (*y w* ; *transgene*/+ ; *qMtn*/*TM6b*) and eclosure rates of transgenic flies were determined. Flies transgenic for wild type MTF-1 reached the expected eclosure frequency of approx. 25 % in a *qMtn* heterozygous or homozygous background, while flies carrying MTF-1 truncations on a *qMtn* background reached less than 5 %.

Figure 5: **A constitutive, acidic activation domain is located between aa 352-540.**

Fusion proteins of the Gal4 DNA-binding domain and subsegments of MTF-1 (A) were tested for their transactivation function with a Gal4-responsive reporter gene (2xGOVEC) in S2 cells (B). Signal levels were normalized to a co-transfected *Actin*-reference. The signal of copper-induced cells transfected with the aa 286-791 Gal4-fusion was set to 1. To test for copper inducibility of these activation domains, cells were treated with 100 μ M CuSO₄ for 16 hrs prior to cell harvest. Segments aa286-791, 286-579 and 352-540 were also tested for their response to cadmium (10 μ M).

Figure 6: **Functional domains of *Drosophila* and human MTF-1.** Schematic overview of the functional domains of *Drosophila* and human MTF-1. Shown are the localization of the zinc fingers, transcriptional activation domains (AD), nuclear localization (NLS) and nuclear export (NES) signals. The metallothionein-like (MT-like) C-terminal domain of *Drosophila* MTF-1 is characterized by a high content of cysteine residues (all cysteines are indicated by red dashes).

Figure 1

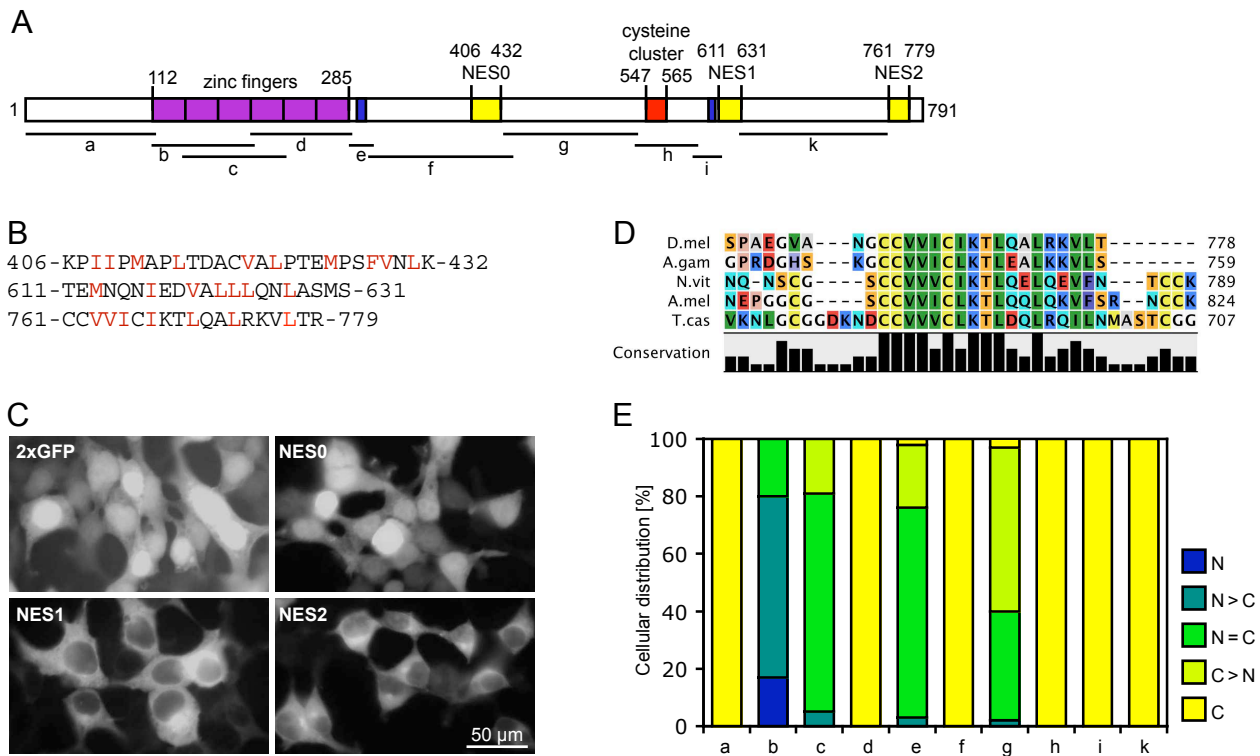
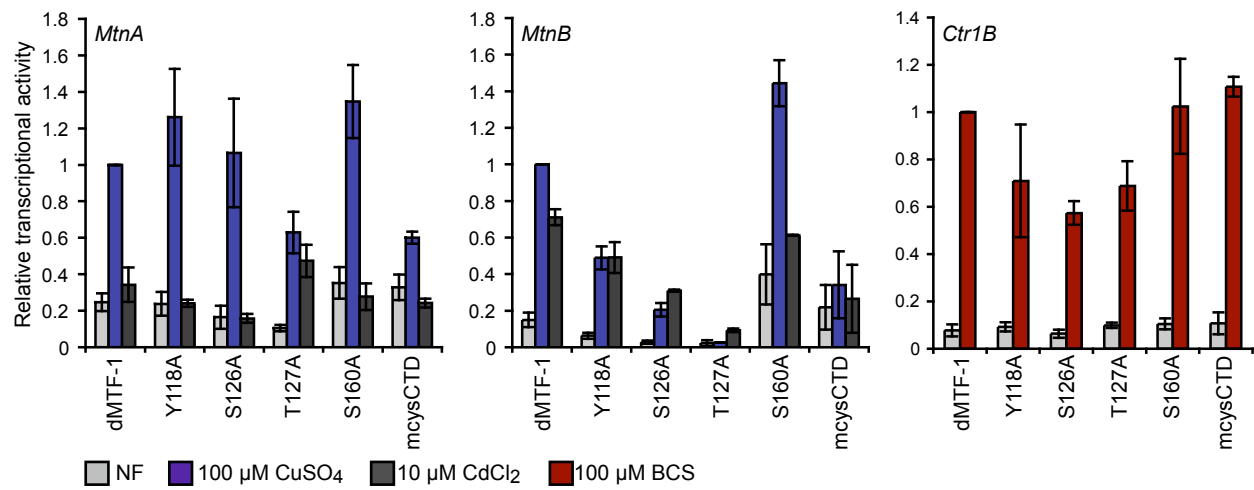


Figure 2

A



B

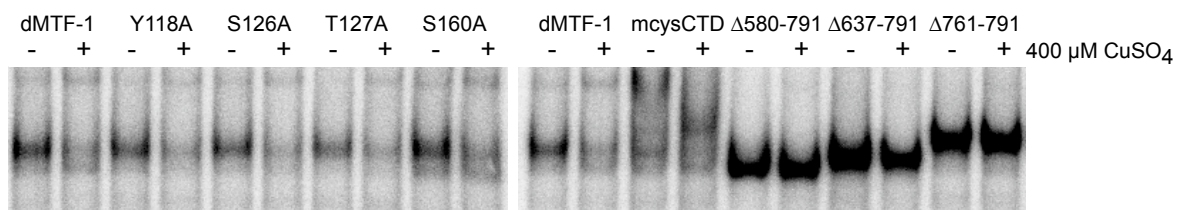


Figure 3

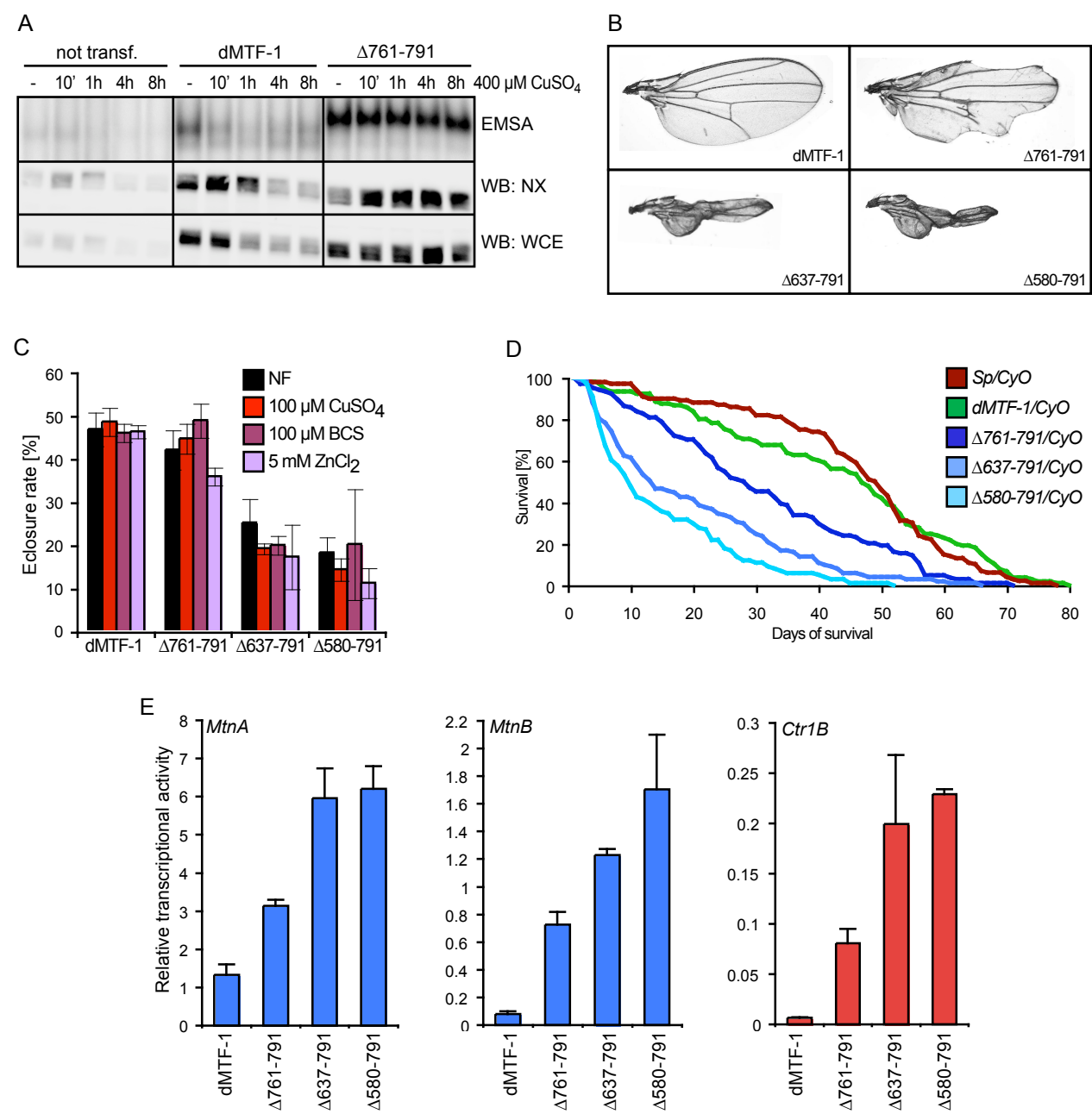


Figure 4

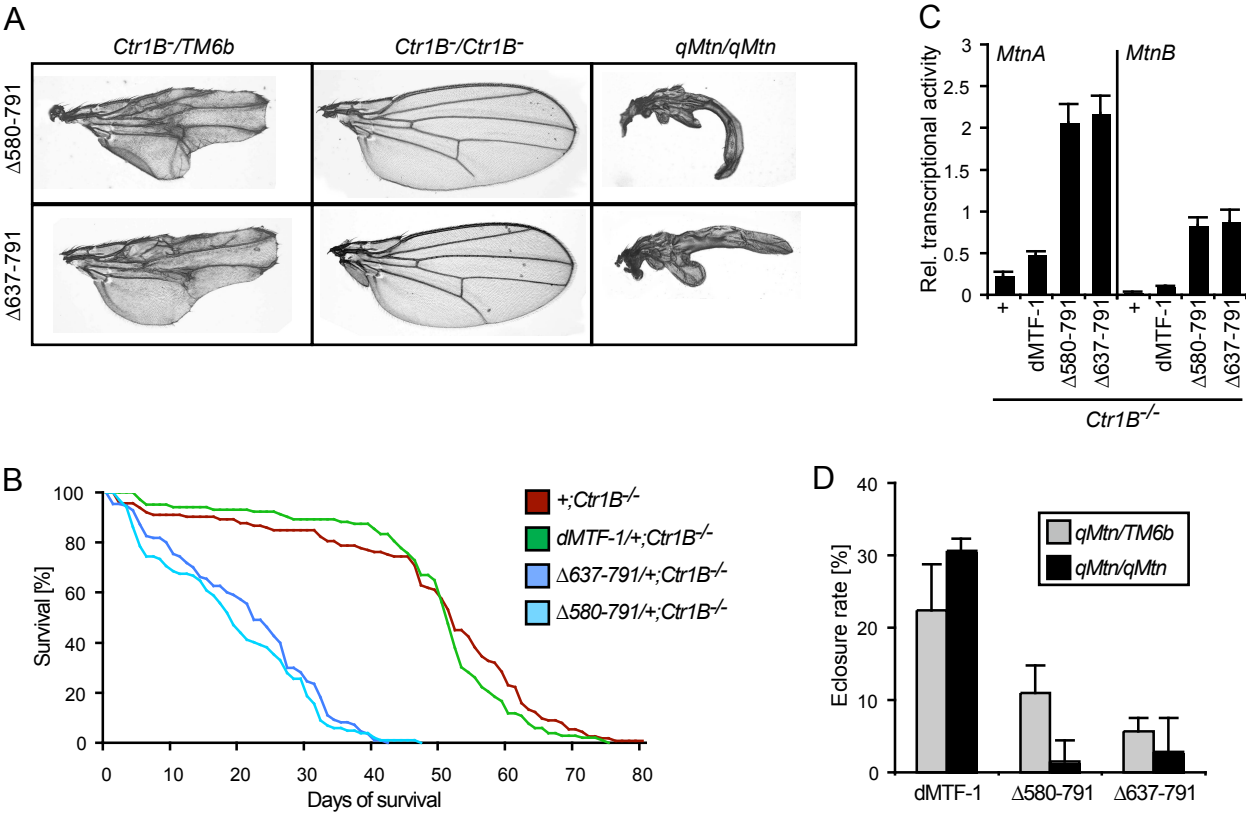
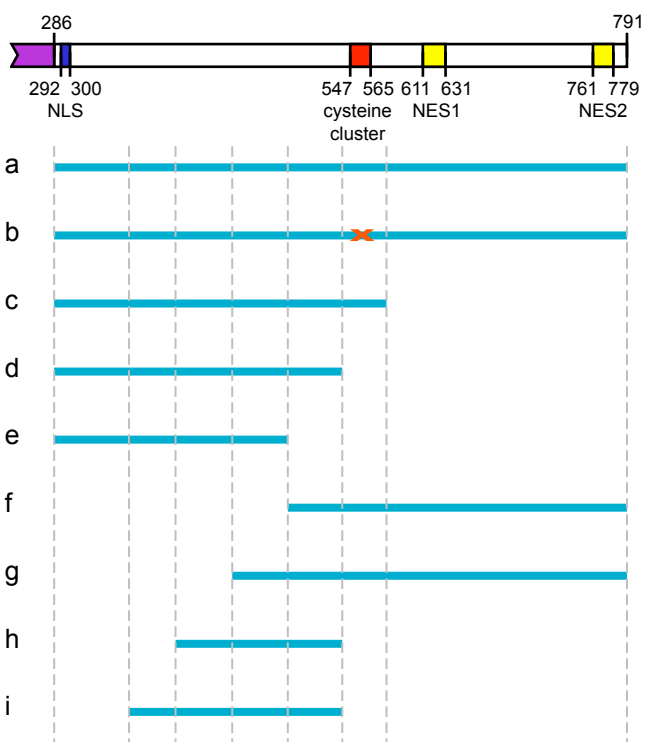


Figure 5

A



B

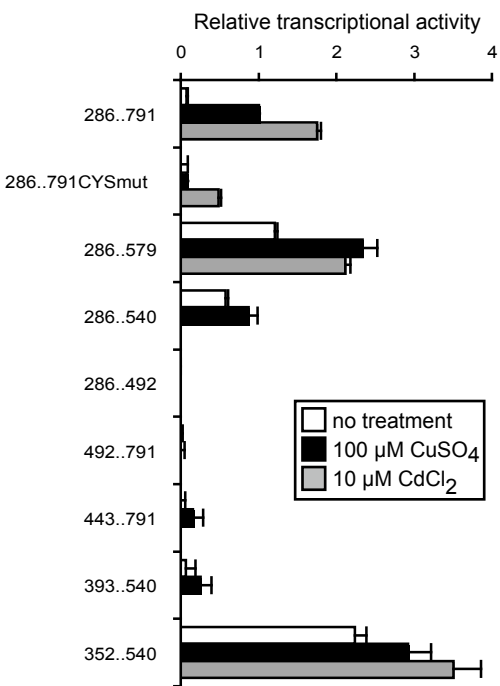


Figure 6

